

Cytokine induction of sol–gel-derived TiO₂ and SiO₂ coatings on metallic substrates after implantation to rat femur

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Abstract: Material surface is a key determinant of host response on implanted biomaterial. Therefore, modification of the implant surface may optimize implant–tissue reactions. Inflammatory reaction is inevitable after biomaterial implantation, but prolonged inflammation may lead to adverse reactions and subsequent implant failure. Proinflammatory activities of cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF- α) are attractive indicators of these processes and ultimately characterize biocompatibility. The objective of the study was to evaluate local cytokine production after implantation of stainless steel 316L (SS) and titanium alloy (Ti6Al4V) biomaterials coated with titanium dioxide (TiO₂) and silica (SiO₂) coatings prepared by sol–gel method. Biomaterials were implanted into rat femur and after 12 weeks, bones were harvested. Bone–implant tissue interface was evaluated; immunohistochemical staining was performed to identify IL-6, TNF- α , and Caspase-1. Histomorphometry (AxioVision Rel. 4.6.3 software) of tissue samples was performed in order to quantify the cytokine levels. Both the oxide coatings on SS and Ti6Al4V significantly reduced cytokine production. However, the lowest cytokine levels were observed in TiO₂ groups. Cytokine content in uncoated groups was lower in Ti6Al4V than in SS, although coating of either metal reduced cytokine production to similar levels. Sol–gel TiO₂ or SiO₂ coatings reduced significantly the production of proinflammatory cytokines by local tissues, irrespective of the material used as a substrate, that is, either Ti6Al4V or SS. This suggests lower inflammatory response, which directly points out improvement of materials' biocompatibility.

Keywords: bone implant, surface modification, sol–gel coatings, inflammation, biomaterial

Introduction

The use of metallic implants is a substantial part of the treatment in orthopedic surgery. Necessary condition of their clinical success is effective osteointegration – bonding between bone and the implant. Immediately after implantation, host tissues react on the biomaterial with acute inflammation, which, within a few days, transforms into chronic phase lasting for months or even years. The dynamics of the latter phase determine the final outcome – long-lasting osteointegration or extensive inflammation leading to implant loosening and subsequent clinical failure.¹ Tissue reaction and inflammatory response on the biomaterial is determined by cell activity (monocytes and macrophages mainly). The activated macrophages not only secrete cytokines to recruit other cell types involved in inflammation, but are also responsible for healing of the implant site.^{1,2} Cytokines are not only known for their regulatory role in inflammatory reactions and bone healing, but also they determine the presence and intensity of the foreign body reaction, and as well as in case of prolonged activity, can negatively affect bone turnover.³

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Therefore, interleukin (IL)-1, IL-6, and tumor necrosis factor- α (TNF- α), with their proinflammatory activity and contribution to osteolytic processes, are attractive indicators to assess the biologic function of biomaterials.^{1,2,4,5}

Implant surface is one of the most important factors regulating the interaction between the biomaterial and the bone tissue.^{6–10} Therefore, techniques of surface modifications are extensively studied in order to improve the clinical performance of biomaterials. The ideal surface is biocompatible, osteoconductive, and osteoinductive, limits corrosion and particle release from the material, and is also mechanically stable with antimicrobial properties.^{11–13}

In the present study, silica (SiO₂) and titanium dioxide (TiO₂) thin films for biomedical applications have been synthesized by nonaqueous sol–gel dip-coating method on stainless steel 316L (SS) and titanium alloy (Ti6Al4V) substrates. Previously, the authors conducted surface studies and in vitro tests of these biomaterials, and demonstrated the potential for applications in biologic environment.^{14–16}

The aim of this study was to evaluate the inflammatory response on stainless steel 316L (SS) and Ti6Al4V biomaterials coated with TiO₂ and SiO₂ sol-gel layers implanted to rat femur.

Materials and methods

Stainless steel 316L (SS) and Ti6Al4V prim-shaped implants, 10 mm long with square base 1×1 mm, were prepared for the in vivo experiments. SiO₂ and TiO₂ thin films were synthesized by nonaqueous sol–gel dip-coating method. Sol–gel synthesis was based on the hydrolysis of alkoxide precursors at room temperature. Tetraethoxyorthosilicate (Sigma-Aldrich Co.) and diethoxydimethylsilane (Sigma-Aldrich Co.) in a molar ratio of 1.79 were used as SiO₂ precursors. As titania precursor, titanium (IV) isopropoxide (Sigma-Aldrich Co.) was used. Directly before coating, the substrates were washed with acetone or dilute HCl, then with

distilled water, and finally with alcohol. Water necessary for hydrolysis was derived as moisture from the atmosphere, according to the method described previously.¹⁷ Synthesis of dioxide coatings, as well as physicochemical assessment, surface analysis, and mechanical studies were conducted as previously published.^{15,16,18,19}

Thirty-two male Wistar rats, with body weight approximately 300 g, aged 3–6 months were used for the experiment. All rats were kept in the same room under standard conditions (12 hours/12 hours of light/darkness period, room temperature 20.5°C±1°C) in separate cages with free access to water and rat chow, without any movement restrictions.

Surgical procedures were carried out under general anesthesia in aseptic conditions. To anesthetize the animals, a mixture of 1 mL ketamine hydrochloride (100 mg/mL) and 0.5 mL xylazine hydrochloride (20 mg/mL) added to 10 mL of 0.9% NaCl was prepared and injected intraperitoneally at a dose of 1 mL/100 mg body weight. The animal leg was shaved, washed in chlorhexidine solution, and positioned and clothed in sterile sheets on the operating table. A curved incision measuring 10–15 mm was made on the anterolateral knee surface; the joint capsule was dissected, incised, and then the patella subluxated, exposing the femur intercondylar fossa (Figure 1A). Femur's medullary canal was opened through the fossa using a 1.2 mm drill and the specimen was positioned in the medullary canal (Figure 1B). Subsequently, the wound was closed and each layer separately sutured: the joint capsule, fascia, subcutaneous tissues, and skin. X-rays were taken just after surgery to confirm proper location of the implants (distal metaphyseal–diaphyseal; Figure 1C). The animals were sacrificed after 12 weeks by administering intraperitoneal injections of pentobarbital (Morbital) at a dose of 2 mL/kg body weight. Distal femurs were dissected and evaluation carried out.

From each animal, three tissue samples were obtained. The samples consisted of 2–3 mm of trabecular bone adjacent

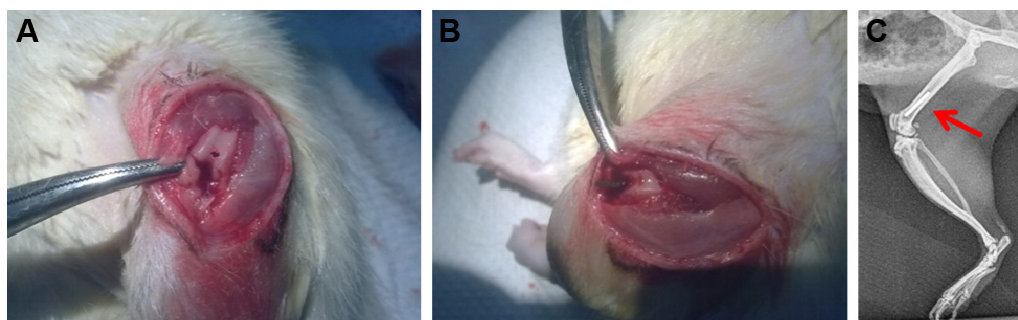


Figure 1 Consecutive stages of implantation: (A) opening of femur medullary canal and (B) implant insertion. (C) Red arrow shows location of the implant on lateral X-ray picture.

to the implant, which originated from three different localizations (proximal, middle, and distal ends of the implant). The animals were randomized and divided into two control groups (uncoated SS 316L implants, n=4; uncoated Ti6Al4V, n=4) and four experimental groups (I. SS coated with SiO₂, n=6; II. SS coated with TiO₂, n=6; III. Ti6Al4V coated with SiO₂, n=6; IV. SS coated with TiO₂, n=6).

All procedures were conducted according to the guidelines for the care and treatment of laboratory animals (EU directive 2010/63/EU), and the study was approved by the local ethics board (The Second Local Bioethical Commission in Wrocław, approval 86/2009).

With a microtome (Zeiss Microm HM 340E), the bones were cut into 3 µm thick sections, dehydrated in xylene and alcohol graded series, and placed on histological slides. After fixation and dehydration, the specimens were incubated in Tris/ethylenediaminetetraacetic acid buffer (pH=9.0) for 20 minutes to carry out heat-induced epitope retrieval. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 5 minutes; then, the samples were briefly rinsed with Tris-buffered saline (TBS) (3×5 minutes). The tissue samples were incubated for 1 hour at room temperature with primary antisera raised against IL-6 (rat, dilution 1:400; Abcam), TNF receptor I (rat, dilution 1:1,000; Abcam), and Caspase-1 (rat, dilution 1:5; Abcam). After subsequent rinsing in TBS (3×5 minutes), the sections were incubated with secondary antibodies (EnVision Systems; Dako) for 1 hour at room temperature. Subsequently, the samples were counterstained with Mayer's hematoxylin, dehydrated in alcohol and xylene series, mounted with permanent mounting medium, and finally covered with a glass coverslip.

Approximately 2 mm layers of tissues adjacent to the implants were evaluated. High-resolution images were taken under a light microscope (Carl Zeiss Axio Imager A1) at 320× magnification, and all images were processed with the same parameters and histomorphometry was performed. AxioVision Rel. 4.6.3 (Carl Zeiss) software was used to identify and mark the tissue containing high concentrations

of cytokines and its total area was estimated in µm². In all specimens, the same cutoff parameters were applied to detect high cytokine levels.

Statistical analysis of independent and dependent variables, with respect to the total area (µm²) of the tissue containing cytokines, was performed using two-way analysis of variance. Based on the results obtained from analysis of variance, two sample *t*-test was used to compare the individual differences between the mean values (µm²) of cytokine-containing areas of the control and experimental groups. Mann–Whitney *U* test or Wilcoxon test was applied for nonparametric analysis. To verify whether the two sample *t*-test can be used, normal distribution of the variables was checked with Shapiro–Wilk test and homogeneity of variance with Brown and Forsythe test. A *P*-value <0.05 was considered statistically significant.

Results

Uncoated SS implants induced much more intense cytokine production (IL-6, Caspase-1, TNF-α) than the SS implants coated with either TiO₂ or SiO₂. It was confirmed with a high significance obtained in statistical analysis (Table 1). Comparison between two tested coatings on SS revealed that TiO₂ induced lower cytokine production than SiO₂ (Figure 2); however, statistical significance was reached only for TNF-α with *P*=0.024 (Caspase-1 *P*=0.374, IL-6 *P*=0.059).

Coated Ti6Al4V with either TiO₂ or SiO₂ significantly decreased the cytokine content in comparison to uncoated Ti6Al4V, except that statistically insignificant result was obtained for Caspase-1 in Ti6Al4V + SiO₂, *P*=0.129 (Figure 2; Table 1).

Comparison of cytokine content in the tissues surrounding coated titanium implants revealed more significant reduction of IL-6 and Caspase-1 in Ti6Al4V + TiO₂. On the contrary, TNF-α was more abundant in Ti6Al4V + TiO₂ than Ti6Al4V + SiO₂. Differences, however, were small and insignificant statistically for IL-6 (*P*=0.0701) and TNF-α (*P*=0.1758), although for Caspase-1, they were more pronounced with a *P*=0.0021 (Figure 2).

Table 1 Mean values (µm²) with standard deviation (±) of cytokine-containing area and statistical analysis of differences between coated and uncoated implants (*P*-values)

Cytokine	SS	SS + SiO ₂	<i>P</i> -value	SS + TiO ₂	<i>P</i> -value	Ti6Al4V	Ti6Al4V + SiO ₂	<i>P</i> -value	Ti6Al4V + TiO ₂	<i>P</i> -value
IL-6	50,115±11,038	14,325±3,242	0.00015	10,241±2,827	0.000065	22,431±5,241	11,498±5,274	0.021	5763±2,211	0.001
Caspase-1	50,561±3,370	15,322±6,647	0.00006	11,550±5,459	0.000003	27,969±1,299	23,639±4,457	0.129	13,479±3,258	0.00006
TNF-α	36,463±3,333	21,542±4,583	0.01933	12,683±4,462	0.000105	28,363±2,463	14,951±4,820	0.002	19,445±5,391	0.020

Abbreviations: IL-6, interleukin-6; SiO₂, silica; SS, stainless steel; Ti6Al4V, titanium alloy; TiO₂, titanium dioxide; TNF-α, tumor necrosis factor-alpha.

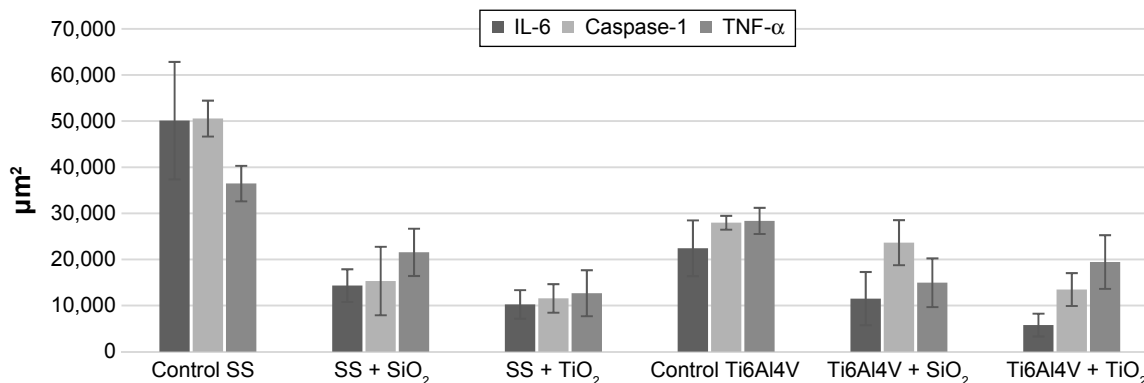


Figure 2 Mean values (µm²) of cytokine-containing tissues in test and control groups.

Abbreviations: IL-6, interleukin-6; SiO₂, silica; SS, stainless steel; Ti6Al4V, titanium alloy; TiO₂, titanium dioxide; TNF-α, tumor necrosis factor-alpha.

The differences in cytokine content between uncoated SS and Ti6Al4V were very distinctive and smaller in Ti6Al4V (IL-6 *P*=0.008, Caspase-1 *P*=0.005, TNF-α *P*=0.015). Coating of either material declined the cytokine production; moreover, it brought them down to similar levels (Figures 2 and 3). The differences between SS- and

Ti6Al4V-coated materials were highly insignificant, and it was proven in the statistical analysis for each cytokine.

Discussion

The extent of tissue reaction after material implantation is either host or implanted material dependent. Regarding

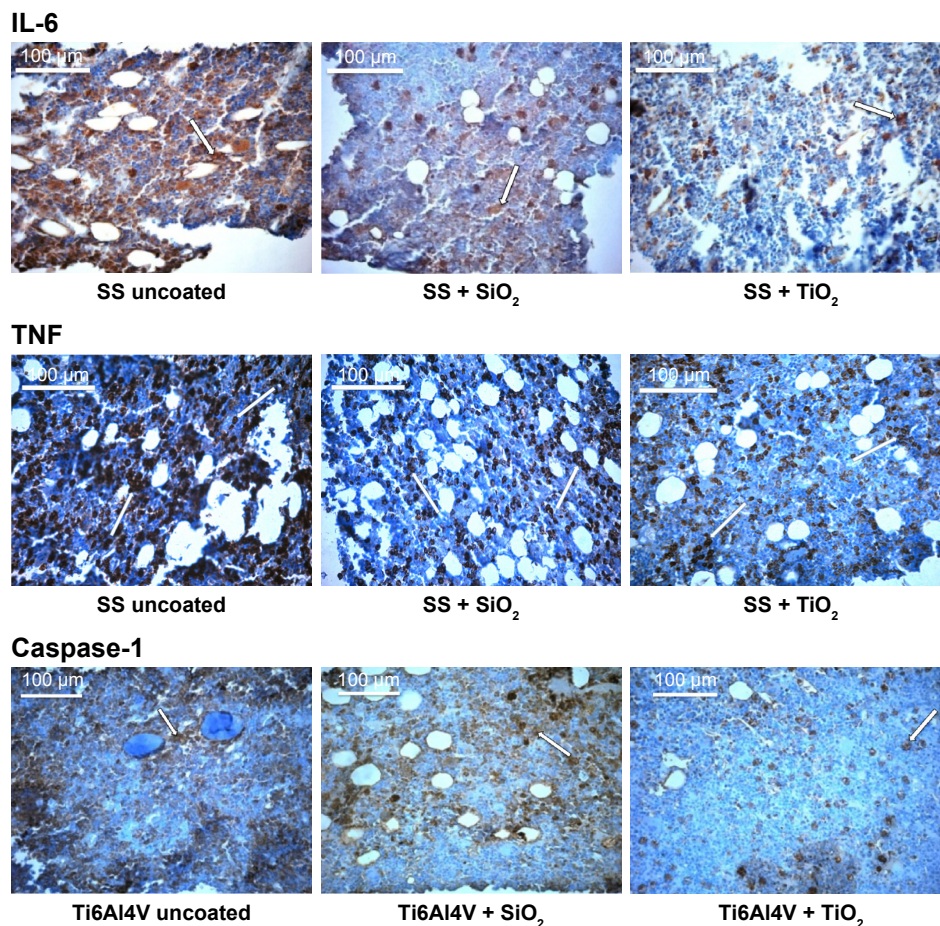


Figure 3 Images from light microscope of cytokine-containing tissue samples after immunohistochemical staining (magnification 8×40).

Note: Dark areas correspond to tissue containing IL-6 or Caspase-1 (white arrows).

Abbreviations: IL-6, interleukin-6; SiO₂, silica; SS, stainless steel; Ti6Al4V, titanium alloy; TiO₂, titanium dioxide; TNF, tumor necrosis factor.

biomaterial features, the most important are the material size, surface topology and chemistry, mechanical forces, and the release of degradation products from the implant.^{3,8–10,20,21} Hence, the interactions between the implant and bone are mostly determined by the biomaterial surface. Therefore, altering the surface and its features is a way to improve implant-tissue interaction.

In this study, the authors tested the biologic interactions of the implants coated with oxides (SiO₂ and titania) obtained with sol-gel method. It was previously demonstrated that sol-gel-derived oxide films were bioactive *in vitro* and *in vivo*, and could induce bone attachment to the metallic materials, which confirmed their suitability as bone implants.^{22–26} The sol-gel method of SiO₂- and titania-based coating synthesis is inexpensive and allows to control the film properties by changing the solution composition or deposition process details (eg, homogenous physicochemical structure, roughness, Young's modulus, etc). It also affects the biomaterial's surface features; topography, roughness, and wettability.^{27,28} With qualities appropriate for cell attachment, like high wettability and surface roughness, bioactivity of the tested sol-gel dioxides was proven and published previously.^{14–19}

An act of implantation of a foreign material into bone always initiates certain cascade of reactions: hematoma, inflammation, and subsequently, either osteointegration or foreign body reaction and implant failure.^{1,3} Nevertheless, particles and debris from the material may induce high levels of proinflammatory cytokines, resulting in persisting inflammation. This may disturb osteointegration, induce FBR, or elicit osteolysis of previously integrated implant.^{2,29,30} The cytokines analyzed in the study – IL-6 and TNF- α – play an important role in osteolysis. Caspase-1 levels were also assessed, since its activity reflects IL-1 levels – Caspase-1 activates precursors of IL-1, another significant contributor to osteolytic process.³¹ It was demonstrated that TNF- α , IL-6, and IL-1 had adverse effects on osteoblastogenesis from mesenchymal stem cells and caused osteoclast-induced bone destruction.^{5,32–34} High concentrations of these proteins were observed in the tissues surrounding loosened endoprosthesis as well as in failed dental implants.^{30,35–38} Hence, local cytokine levels may be considered as an indicator of biomaterial compatibility and also its performance. Thus, creating biomaterial that results in the lowest possible tissue cytokine secretion might improve its clinical performance.

In this study, the authors observed substantial drop in cytokine levels after coating with Ti6Al4V and SS materials. More considerable decrease was noticed in SS-based

materials than Ti6Al4V-based materials, because of good biocompatibility of titanium and its alloys and poor biocompatibility of SS. Coating of either material declined cytokine production and brought it down to similar levels (Figure 3), even enhancing the biocompatibility of coated SS above that of uncoated Ti6Al4V levels, particularly of SS + TiO₂ (Figures 2 and 3). Thus, on modification of SS – an inexpensive metal with low biocompatibility, low corrosion resistance, containing allergenic ingredients (eg, nickel) but with excellent strength, we obtained a biomaterial with high biocompatibility, concomitantly maintaining its mechanical properties. Such a compound can be used for internal fixation of long bones as well as in the operative treatment of spinal deformity, since the rods used for correction and spine fixation in spinal surgery should be of high strength and stiffness.

To our knowledge, there is no report on alteration in cytokine levels on sol-gel TiO₂ or SiO₂ coatings, although reduction of inflammatory response after coating the implants with sol-gel oxide layers was reported by other authors.^{39–42} Data presented in this paper suggest better cytokine reduction of TiO₂ coatings on Ti6Al4V and SS, which was consistent to other authors' findings on inflammatory response. There are no reports of comparative analysis between these layers in the literature available. It seems that superiority of anti-inflammatory activity of TiO₂ can be related to its additional antioxidant properties. According to the study of Contreras et al, titanium oxide reduces the level of reactive oxygen species (free radicals), both neutrophilic and of chemical origin.⁴² Another contribution may be the fact that SiO₂ layer has inferior stability than TiO₂, and it is a partially degradable material, releasing the particles to the environment.⁴³

The strength of the study is that the analysis was conducted in bone tissue, an environment of the final implant, thus providing information concerned with the target tissue.

Lack of proper estimation of the concentration of the given cytokines in this study may be considered as a weakness; however, the method presented is simple and provides clear and reliable data. Common methods to assess the level of cytokine include either direct staining for substance concentration or molecular methods based on quantification of cytokine mRNA. To evaluate the protein concentration, exudative fluid is needed; but the methodology to obtain it is complicated and impedes assessing other parameters of implant integration. It was also demonstrated that the amount of mRNA was not always proportional to proinflammatory cytokine activity and was not equal to the observed inflammatory reaction.⁴⁴

Conclusion

Sol-gel TiO₂ or SiO₂ coatings reduced significantly production of proinflammatory cytokines by the local tissues, irrespective of the material used as a substrate, that is, either Ti6Al4V or stainless steel 316L. This suggests lower inflammatory response, which directly points out improvement of materials' biocompatibility. SS, an inexpensive metal popular in orthopedic and dental surgery, is known for possessing desired mechanical properties, but is of low biocompatibility. After oxide sol-gel coating, it is converted to a biocompatible biomaterial, which widens the range of its clinical applications.

Disclosure

The authors report no conflicts of interest in this work.

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